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Short communication

"HOOF-Print" genotyping and haplotype inference discriminates among *Brucella* spp. isolates from a small spatial scale

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ABSTRACT

We demonstrate that the "HOOF-Print" assay provides high power to discriminate among *Brucella* isolates collected on a small spatial scale (within Portugal). Additionally, we illustrate how haplotype identification using non-random association among markers allows resolution of *B. melitensis* biovars (1 and 3). We recommend that future studies use haplotype identification when analyzing multilocus population genetic data to help discriminate among microbial isolates such as *Brucella*.

1. Introduction

Brucellosis is an important zoonosis with worldwide distribution and high clinical morbidity. Bacteria from the genus *Brucella* can infect a variety of hosts and are responsible for significant economic losses in livestock industries and serious public health problems in humans. Transmission to man can occur through many routes: foodborne, occupational, recreational and potentially through bioterrorism (Godfroid et al., 2005). Brucellosis continues to be a major problem in the Mediterranean Basin, Middle East, Latin America, Asia and Africa (Godfroid et al., 2005; Pappas et al., 2006). Portugal, like other European Union countries, employs specific regulations and measures to eradicate the disease. Regardless of the huge efforts to eliminate it, human cases still occur frequently in the country (16.1 cases per million, Direcção Geral de Saúde, Divisão de Epidemiologia, 2006) and the prevalence in cattle and small ruminants herds is 0.25 and 0.70%, respectively (Direcção Geral de Veterinária, 2007).

Control of brucellosis, particularly in the final stages of an eradication program, requires a rigorous program for surveillance and highly discriminatory methods for characterizing an outbreak strain, which can be used in trace back studies to determine the original source of infection and its routes of transmission. Conventional methods for subtyping of *Brucella* strains into species and biovars have some shortcomings, particularly, in small geographical regions where few biovars tend to predominate (e.g., most *B. melitensis* isolated in Portugal belong to biovars 1 and 3; LNIV, National Laboratory for Veterinary Research, unpublished data). Also, classification of *Brucella* relies on a large array of phenotypic tests that are prone to misinterpretation or inaccuracy (Banai et al., 1990; Ewalt and Forbes, 1987).

Moreover, because of the high genetic homology among bacteria of the genus *Brucella*, there is a demand for the development and validation of highly polymorphic markers to increase sensitivity and resolving power. Recently, Bricker et al.

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(2003) identified a set of eight VNTRs (variable number tandem repeats) that were integrated into an assay named "HOOF-Prints", (Hypervariable Octameric Oligonucleotide Finger Prints). The assay targets genetic regions that show a high degree of intraspecific diversity. These authors demonstrated that HOOF-printing allows high-resolution discrimination among *Brucella* isolates, an important tool to significantly improve disease control. When applied to *B. abortus* strains from across North America (Bricker and Ewalt, 2005), this technique satisfies the established requirements for bacterial strain typing methods recommended by European Study Group for Epidemiological Markers (ESGEM; Struelens, 1996). In 2006, two other groups (Le Fleche et al., 2006; Whatmore et al., 2006) tested different panels of multiple locus VNTRs (including some of the HOOF-Print markers) in larger collections of *Brucella* isolates and confirmed that the assays have potential as epidemiological tools.

Although various sets of VNTRs have been studied, none of the approaches used thus far were able to distinguish all the biovars of *Brucella* spp. (e.g., Le Fleche et al., 2006; Whatmore et al., 2006). Therefore there is a great need to develop and evaluate not only new molecular techniques, but also to use new and existing computational/statistical methods that could help to increase the power of intraspecific discrimination (e.g., biovar within European countries).

We employed the "HOOF-Print" assay to genotype isolates of *Brucella* spp. obtained from different hosts and from a relatively small geographic area (Portugal) and analyzed the dataset using non-random association (Gametic disequilibrium, GD) testing and haplotype identification (Excoffier et al., 2005; http://lgb.unige.ch/arlequin). Gametic Disequilibrium, like Linkage Disequilibrium (LD), tests for non-random association between alleles at different loci. Disequilibrium (non-random association) occurs when alleles at different loci occur together more often than can be accounted for by chance. LD refers to alleles at loci that are physically close on the DNA strand but GD refers to any loci—including those that are not linked physically. GD measures statistical association (non-random), not physical association. We found linkage groups or allele combinations that are statistically associated (see below) and conducted the analysis according to this. Once linkage groups (haplotypes) were found, analysis of evolutionary relationships among the allele combinations was performed using the phylogenetic network algorithm contained in the software NETWORK, version 4.2.0.1 (http://www.fluxus-engineering.com; Bandelt et al., 1999). The median-joining algorithm included in this software has been demonstrated to be the most appropriate algorithm to handle multiple state data such as ours (i.e., VNTRs). A NETWORK analysis was conducted because, in addition to the inference of the phylogenetic relationships among haplotypes, it facilitates visual representation of the frequencies of each haplotype. Thus, it allows a more complete identification and interpretation of patterns in our dataset, especially when compared to the classical phylogenetic trees used in most studies.

Isolates used in this study were obtained from LNIV, as part of National Eradication Program for this disease. Each collected tissue (lymph nodes, spleen, liver, uterus and mammary gland) was homogenized in sterile phosphate-buffered saline (PBS pH 7.2) and the homogenate seeded on duplicated plates of modified Farrell medium. Plates were incubated up to ten days at 37 °C in normal and supplemented (5% of CO_2) atmosphere. Species and biovar identification were performed according to Alton et al. (1988). In a total, 71 *Brucella* isolates (51 *B. melitensis* and 20 *B. abortus*) were randomly selected to represent different hosts and different locations across Portugal (Additional File 1). Total genomic DNA was extracted using a commercial kit (Puregene, Gentra Systems, USA). HOOF-Print genotyping was performed as described by Bricker et al. (2003). All VNTR loci were amplified in independent PCR reactions under the previously described conditions (Bricker et al., 2003). Each 15-μl reaction mixture consisted of 0.6 units of GoTaq® Flexi DNA polymerase (Promega), 1× PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTP's, 0.2 μM forward and reverse primers and approximately 10 ng of DNA. One of the primer pairs was labelled with Cy5 on the 5′-end for detection in an ALFexpress DNA sequencer. The amplicon sizes were calculated from co-migrating size markers in each lane, by the ALFwin Fragment Analyzer (v.1.02 – Amersham Biosciences) and scored by two independent researchers. Some isolates were tested by an independent laboratory (NADC, USDA, Iowa) to confirm and improve the quality of our data. These isolates were also typed with the HOOF-Print protocol, and the amplicon DNAs were sequenced to validate the results.

Typeability and reproducibility were estimated as suggested by the ESGEM (Struelens, 1996). Genetic diversity was quantified by the Simpson's diversity index (Simpson, 1949) and the Hunter–Gaston discrimination index (HGDI; Hunter and Gaston, 1988) via the online tool V-DICE available at the HPA website (http://www.hpa-bioinfotools.org.uk/cgi-bin/DICI/DICI.pl). The discriminatory power of HOOF-Print genotyping was determined for all isolates and for each species. Allelic richness was estimated using rarefaction with the program HP-RARE 1.0 (Kalinowski, 2005). The number of alleles in a sample is a fundamental measure of genetic diversity, however, this diversity measure can be difficult to use because large samples are expected to contain more alleles than small samples. The statistical technique of rarefaction corrects for this sampling disparity (Kalinowski, 2005).

Among the 71 *Brucella* isolates representing Portuguese diversity (Additional File 1), we found quite good typeability results. Typeability is the ability of getting a measurable and unambiguous result from an experiment. We estimate for all targets tested, a typeability of 98% for *B. abortus* and 95% for *B. melitensis* (157/160 and 386/408, respectively). For complete multilocus genotypes, we have a lower typeability, as expected (i.e. 85% for *B. abortus* and 82% for *B. melitensis*). The calculated reproducibility of our results ($R = 0.983$ at the locus level, and $R = 0.967$ at the composite fingerprint level) was similar to that reported by Bricker and Ewalt (2005) and meets the recommended limit ($P \geq 0.95$).

Diversity Indices (HGDI and Simpson's Index) were reasonably high despite our relatively small geographic study area (Table 1). HGDI ranged from 0 to 0.95 when considering all 71 isolates (Table 1). Allelic diversity (e.g. alleles per locus) was comparable to results reported for samples from across the world (Le Fleche et al., 2006; Whatmore et al., 2006) and ranged from 1 to 23 among loci (Table 1). Equivalent results were obtained with Simpson's Index. The Portuguese isolates have more alleles at Locus 1 and 4 than was reported for isolates from across the world (Le Fleche et al., 2006; Whatmore et al., 2006). Allelic richness for both *Brucella* species was similar (approximately six alleles per locus). These results are highly encouraging and important given the relatively small spatial scale of this study (within Portugal).

The most discriminatory loci were Locus-7 in *B. abortus* and Locus-1 in *B. melitensis* with values of 0.94 and 0.90, respectively. The least discriminative locus was Locus-6 for *B. abortus* (HGDI = 0.56) and Locus-3 for *B. melitensis* (HGDI = 0). To advance the understanding of the general discriminatory power of each VNTR we compared our diversity indices with those reported by other authors (Additional File 2). Our results are similar to those described by Bricker et al. (2003) and Bricker and Ewalt (2005) but we found a considerably higher diversity at Locus-5 and 8 for *B. abortus* (e.g. HGDI of 0.912 and 0.600 respectively, compared to 0.04 and 0.0). Most of the diversity at Locus-8 comes from the *B.*

Table 1
Diversity indices (Hunter and Gaston Diversity Index (HGDI) and Simpson's Diversity Index (Simpson's DI)); number of alleles (Na); number of individuals (Ni); and allele lengths for each *Brucella* spp. and VNTR locus.

Locus	Brucella spp.					*Brucella abortus*					*Brucella melitensis*				
	Ni	Na	Range (bp)	Diversity Index		Ni	Na	Range (bp)	Diversity Index		Ni	Na	Range (bp)	Diversity Index	
				HGDI	Simpson's DI				HGDI	Simpson's DI				HGDI	Simpson's DI
LOCUS-1	69	23	91–211	0.945	0.931	20	11	92–180	0.911	0.865	49	13	91–211	0.904	0.885
LOCUS-2	71	5	101–133	0.648	0.639	20	3	101–117	0.647	0.615	51	5	101–133	0.572	0.561
LOCUS-3	71	5	128–176	0.276	0.273	20	5	128–176	0.663	0.630	51	1	128	–	–
LOCUS-4	69	20	102–229	0.933	0.919	20	7	102–158	0.816	0.775	49	13	109–229	0.895	0.877
LOCUS-5	62	14	139–251	0.908	0.893	19	8	139–203	0.912	0.864	43	12	155–251	0.903	0.882
LOCUS-6	69	9	150–19	0.867	0.854	20	3	159–175	0.563	0.535	49	6	150–190	0.805	0.789
LOCUS-7	65	12	94–206	0.890	0.877	18	10	102–206	0.935	0.883	47	9	94–166	0.868	0.849
LOCUS-8	67	8	139–180	0.640	0.631	20	4	140–180	0.600	0.570	47	4	139–179	0.335	0.328
Total		88					53					63			

abortus biovar-3 isolates. This biovar is not found in the USA, which explains the difference between our data and the data originally published by Bricker. Among the *B. melitensis* samples analyzed, VNTR Locus-3 contained a single allele, which is similar to the findings by Bricker et al. (2003).

As the genomes of the *Brucella* species studied have two chromosomes, the probability of non-random associations between loci (GD) is high. Indeed, it is widely accepted that GD between loci can bias phylogenetic and population genetics analysis (Vernez et al., 2005). Thus, we tested our data for GD and found two main linkage groups (i.e., loci with non-random association between pairs of alleles) of five loci in *B. melitensis* (locus 1 + 2 + 4 + 5 + 6 and 4 + 5 + 6 + 7 + 8) and three groups of two loci in *B. abortus* (locus 2 + 8; 3 + 4; and 5 + 6) ($P < 0.05$). Therefore, we combined the genotypes into these groups and analyzed data using haplotypes and not individual loci.

An important finding is that the phylogenetic network reconstruction using haplotypes clearly separates biovar 1 and 3 (*B. melitensis*) when "HOOF-Print" locus-specific genotypes were combined into multilocus haplotypes (Fig. 1). Important also, is to point out that distinction between biovars 1 and 3 of *B. melitensis* was not possible using all loci as independent. It is interesting to note that when applying the same haplotype identification methodology to the available data reported by Whatmore et al. (2006) (Portuguese strains) we obtained similar results (data not shown) suggesting that haplotype identification can improve biovar identification.

In summary, we could discriminate between *Brucella melitensis* biovars from Portugal only when using haplotype reconstruction, which identifies statistically associated markers as a single multilocus block. This is to our knowledge the first study that tests for improved discrimination power using haplotypes. Identification of haplotypes is widely conducted and recommended in phylogenetics and population genetics and merits further investigation and application in microbial discrimination studies. Finally, the high polymorphism observed in our sample

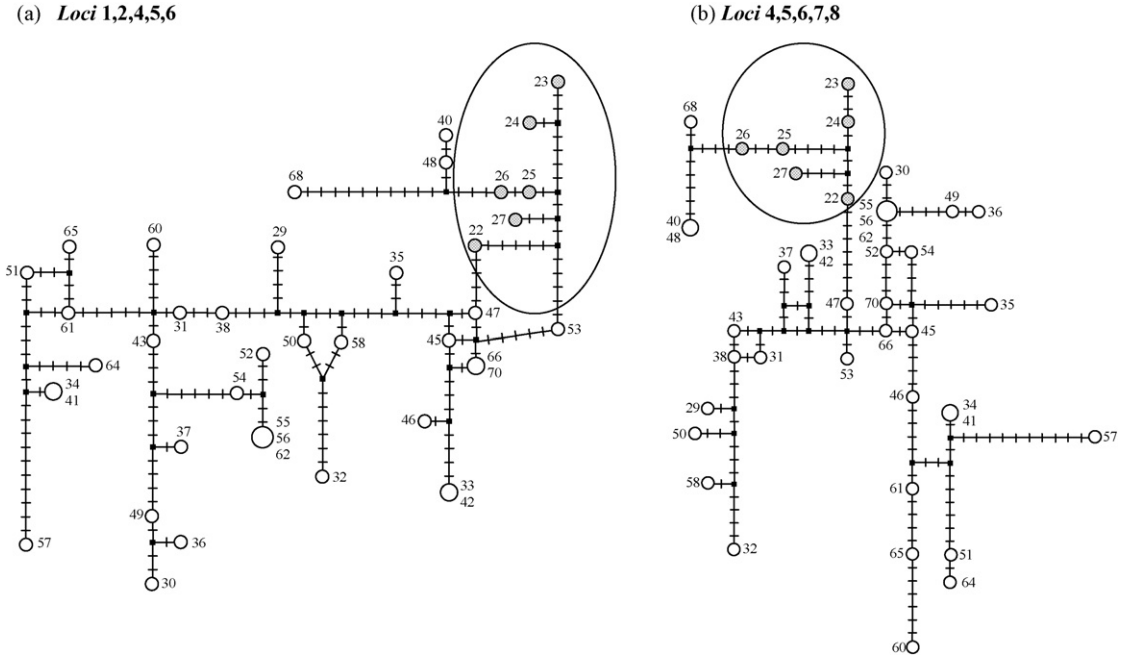

Fig. 1. Phylogenetic networks of haplotypes from two linkage groups (a and b) showing that biovars 1 and 3 can be distinguished (● *Brucella melitensis* biovar 1, ○ *Brucella melitensis* biovar 3). *Note*: Samples number has correspondence with Additional File 1.

confirms the usefulness of the HOOF-print loci to discriminate *Brucella* species, biovars, and isolates across a relatively small geographic scale such as Portugal.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2008.10.007.

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